Effects of glucose and hormones on UCPs gene expression in rat epididymal adipocytes

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Summary

Uncoupling proteins (UCPs) control body temperature and energy balance. We report here the glucose and hormone regulation of the UCP-2 and -3 mRNA and protein expression in primary cultured epididymal adipocytes of rat. The UCP-2 and -3 mRNA and protein induction in the adipocytes reached maximal levels at 4 h in the presence of glucose with or without insulin. Moreover, the UCPs induction was accelerated by triiodothyronine (T3) or epinephrine, and reached a maximum at 2 h. It appeared that the induction of UCPs mRNA and protein was rapid. UCP-2 mRNA expression was more markedly increased by glucose, unsaturated fatty acids, insulin and T3 than UCP-3 mRNA expression. UCP-3 expression was more markedly increased by epinephrine than by T3. The protein expression of the UCPs was induced by glucose and the hormones nearly parallel to the UCP mRNA expression. It is suggested that theUCP-2 expression appears to be stimulated by energy sources such as glucose and fat, and by regulators of thermogenesis and basal metabolic rate such as T3 and insulin, in contrast to UCP-3 expression.

Introduction

Visceral fat accumulation by over nutrition may play an important role in the development of the metabolic syndrome, such as hyperlipidemia, hypertension, and glucose intolerance, leading to advanced atherosclerosis[1)] and may account for the changes in peripheral and hepatic insulin sensitivity seen with obesity[2–4)]. Visceral fat is composed of several adipose depots including mesenteric, epididymal white adipose tissue and perirenal depots.

It is well known that fatty acid synthesis is regulated in response to the nutritional and hormonal states of animals. We previously found that lipogenic enzyme (related to fatty acid synthesis) gene expression in rat liver and epididymal adipose tissue is elevated by a fat-free, high-carbohydrate diet and is suppressed by polyunsaturated fat[5)]. In the present experiments, the nutritional and hormonal regulation of energy consumptions are investigated.

Uncoupling proteins (UCPs) are inner mitochondrial membrane transporters which uncouple to

substrate oxidation for ATP synthesis, converting fuel into heat[6]. UCP families are involved in the control of body temperature and the regulation of energy balance. UCP-1 is expressed exclusively in brown adipose tissue[7]. UCP-2 is expressed in a wide variety of tissues, and highly expressed in white adipose tissue[8–10]. UCP-3 is expressed at high levels in skeletal muscle as well as brown adipose tissue[11]. The gene expression of the UCPs appears to be differentially regulated[12–14].

Triiodothyronine (T3)-treatment stimulates UCP-2, -3 mRNA expression in human skeletal muscle[15], and UCP-2 mRNA expression in rat white and brown adipose tissues and skeletal muscle[16]. Norepinephrine induces UCP expression through a beta adrenergic mechanism at the transcriptional level in brown adipose tissue[17, 18]. Fatty acids have been reported to act as transcriptional regulators of the expression of lipid-related genes in adipose cells[19]. Polyunsaturated fatty acids and monounsaturated fatty acids dramatically up-regulate UCP-2 mRNA levels in 3 T3-L 1 preadipocytes[20]. Krauss *et al.* reviewed the still obscure roles of the UCP homologues in normal physiology, together with their emerging role in pathophysiology, and pointed out the exciting potential for further investigation[21]. As most studies on the function of UCPs in epididymal adipose tissue have been performed in vivo, the direct effects of the hormones and nutrients on UCP mRNA and protein expression are still not clear. Primary adipocytes should be a useful model system for analyzing the regulatory mechanisms of UCP expression and UCP functions. In the present study, we evaluated the direct effects of insulin, glucose, their correlation, T3 and epinephrine on UCP mRNAs and proteins in rat epididymal adipocytes.

Materials and Methods

Materials. [α-^{32}P]dCTP (111 TBq/mmol) was purchased from MP Biomedicals, Inc. (CA, USA). Restriction endonuclease and a DNA sequencing kit were purchased from Takara Shuzo (Shiga, Japan). William's medium E was purchased from Sigma Aldrich (St. Louis, MO, USA). Nylon filter (Hybond N) was purchased from Amersham (Buckinghamshire, UK). Most other reagents were obtained from Sigma and Wako (Osaka, Japan). Antibody against UCP-2 from Biolegend (CA, USA) and antibody against UCP-3 were obtained from Santa Cruz (CA, USA).

Cell culture. Wistar rats (Japan SLC, Sizuoka) maintained on a stock diet (MF, Oriental Yeast Co.) were starved for 16 h before they were killed. Isolated adipocytes were prepared from epididymal fat pads of rats by collagenase digestion[22] and 5×10^5 cells were placed into polystyrene tubes (BD Falcon, NU, USA). Subsequently, the cells were cultured for 8 h in experimental media (with 100 mg/ml streptomycin, 100 units/ml penicillin, 26 mM sodium carbonate, 2 mM glutamate and 1 μM dexamethasone) containing 0, 5, 10 or 20 mM glucose with or without 0.1 μM insulin. When included, 0.1 mM fatty acid, 0.1 μM T3 or 1 μM epinephrine was added. The cells were cultured in a humidified

chamber at 37℃ under 5% CO_2 in air. Cells were harvested at different periods of time, as indicated the figure legends.

Preparation of RNA and dot blot hybridization. Total cellular RNA from adipocytes was isolated by the method of acid guanidum thiocyanate-phenol-chloroform extraction[23]. Dot blot hybridization of total cellular RNA was performed as described previously[24]. UCP-2, -3 cDNA fragments spanning nucleotides +100 to +871 and +100 to +868, respectively, were cloned from rat adipose tissue by reverse transcription and polymerase chain reaction amplification according to Ref. 11. The genomic clone of rat rRNA was obtained from Japanese Cancer Research Resources Bank. A BamHI/EcoRI fragment about 1 Kb long of this clone was isolated and used as a probe for 18 S rRNA. The probes were labeled using a BcaBEST-labeling kit (Takara Shuzo) and [α-^{32}P]-dCTP. To measure the mRNA levels, the total RNA (10-30 μg) was denatured with formamide, spotted on a nylon filter and then hybridized with ^{32}P-labeled cDNA as described previously[24]. The relative densities of hybridization signals were determined by scanning the autoradiograms at 525 nm and normalized by the values of the 18 S rRNA. The mRNA levels were measured by the dot blot hybridization method, and many were confirmed by Northern blot analysis.

Western blotting. Proteins extracted from adipocytes were separated by 8% SDS-polyacrylamide gel electrophoresis according to Laemmli[25] and blotted onto nitrocellulose using a Bio-Rad (Tokyo, Japan) semidry transfer apparatus according to the manufacturer's instructions. The membranes were blocked with 5% membrane blocking agent (Amersham) in Tris-buffer and saline containing 0.1% Tween 20 (blocking solution), and then incubated with UCP-2 or -3 antibody followed by horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham) in blocking solution. Bound antibody was detected by enhanced chemiluminescence following the manufacturer's directions (Amersham).

Statistical analysis. The all results were expressed as the mean±SEM of four or six of different experiments as indicated in tables and figures. One- or two-way ANOVA was followed by inspection of all differences between pairs of means using the least significant difference test[26]. All statistical analyses were performed using the Stat View J-5.0 program. Differences were considered significant at $P < 0.05$.

Results

Insulin dose-dependent regulation of UCP mRNA and protein in rat epididymal adipocytes

The dose-dependent regulation of UCP-2 and -3 mRNA and protein expression by insulin in isolated epididymal adipocytes of rats was measured. The medium contained 5 mM glucose, which is a physiological concentration. The UCP-2 and -3 mRNA expression increased dose-dependently with

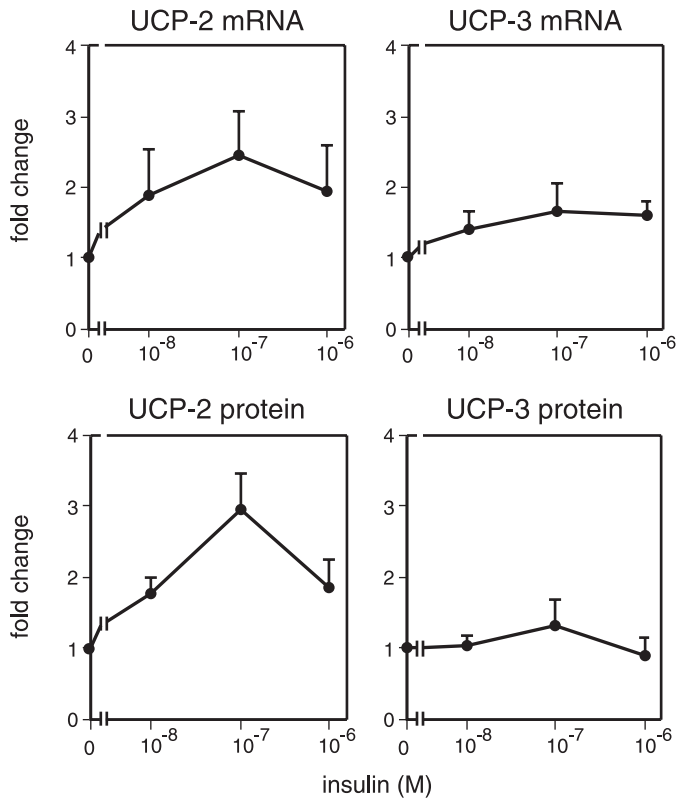

Fig. 1. Insulin dose-dependent regulation of UCP mRNA and protein. The epididymal adipocytes were cultured for 4 h in medium containing 5 mM glucose and 0-10^{-6} M insulin, and the UCP mRNA and protein levels were measured. The relative mRNA and protein levels were normalized by those with no addition. Means±SEM of four different experiments. One-way ANOVA for UCP-2 and UCP-3 : Insulin concentration, $P < 0.05$.

insulin (10^{-8}M–10^{-6}M) (Fig. 1). For the UCP-2 and -3, the maximal increases were observed at 10^{-7}M insulin. The expression of UCP-2 and -3 proteins by insulin was about parallel to their mRNA expression.

Glucose dose-dependent regulation of UCP mRNA and protein in rat epididymal adipocytes

The effects of glucose with or without insulin on the expression of UCPs mRNA and protein are shown in Fig. 2. The addition of glucose to the culture medium caused marked dose-dependent increases in the expression of UCP-2 and -3 mRNAs. The UCP-2 mRNA expression was more markedly induced by the presence of both glucose and insulin than by glucose alone in the culture medium. The expression of UCP-2 and -3 proteins by insulin was about parallel to their mRNA expression, whereas the protein expressions stimulated by insulin were not so large as the mRNA expressions.

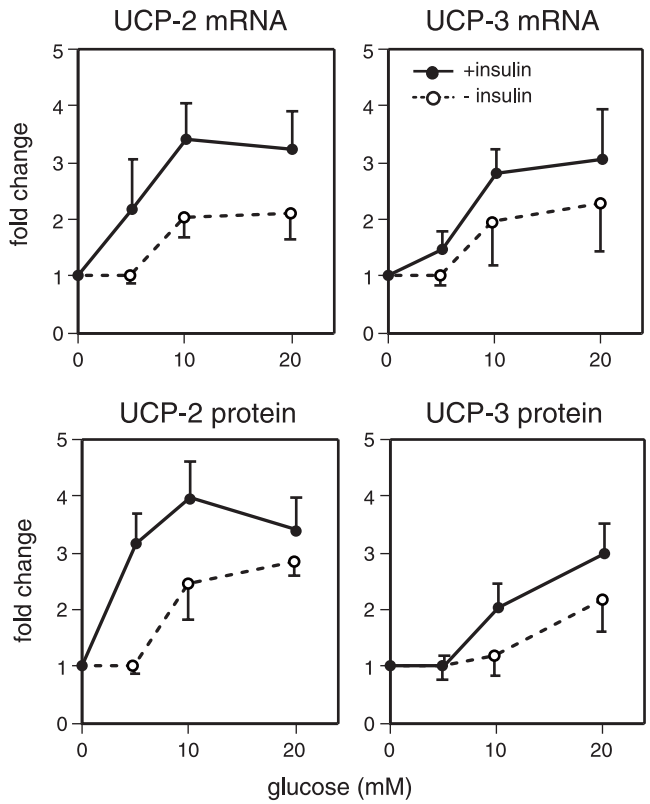

Fig. 2. Glucose dose-dependent regulation of UCP mRNA and protein. The rat epididymal adipocytes were cultured for 4 h in medium containing 0-20 mM glucose with or without 0.1 μM insulin, and the UCP mRNA and protein levels were measured. The relative mRNA and protein levels were normalized by those with no addition. Means±SEM of six different experiments. Two-way ANOVA for UCP-2, -3 mRNA : glucose(Glu), $P<0.001$, Ins (insulin), $P<0.001$; UCP-2, -3 protein : Glu, $P<0.001$, Ins, $P<0.05$, respectively.

Time courses of the UCP mRNA and protein expression stimulated with glucose and insulin in rat epididymal adipocytes

The time courses of UCP-2, -3 mRNA and protein expression in isolated epididymal adipocytes were measured after addition of 20 mM glucose with or without 0.1 μM insulin. The mRNA expression of UCPs reached a maximal level at about 4 h after the treatment, and then decreased (Fig. 3, upper panel). The UCP mRNA expression levels were elevated 3–4 fold or more at the peak. The mRNA expression of UCP-2 and -3 was more markedly induced in the presence of both glucose and insulin than in the presence of glucose alone in the culture medium. The time-courses of UCP-2, -3 protein expressions in epididymal adipocytes are also shown in Fig. 3, lower panel. The protein expression levels of UCPs were elevated and reached a maximum at 4 h, similarly to the mRNA. Thus, the UCP-2 and -3 protein expression was induced by glucose alone, and was more markedly induced in the presence of both glucose and insulin. The UCP protein expression was parallel to the UCP mRNA induction.

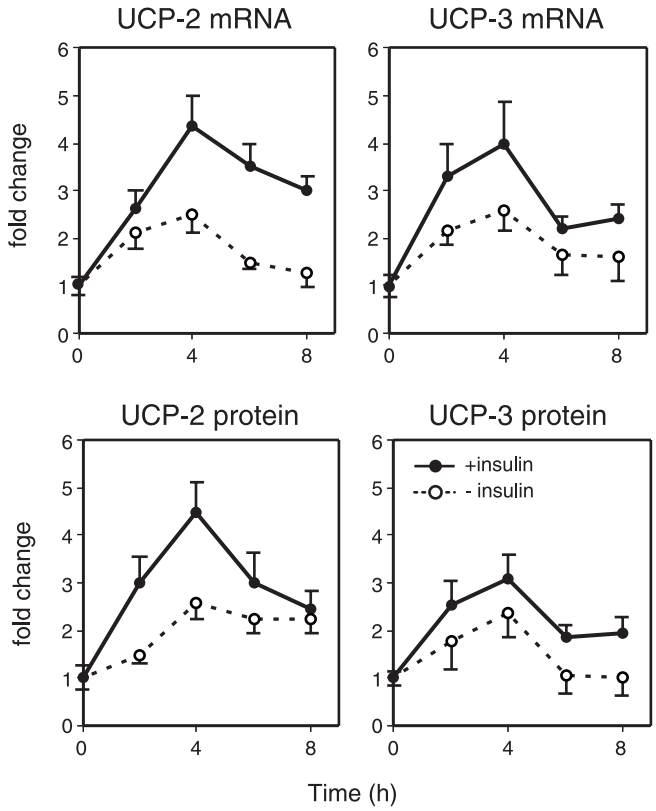

Fig. 3. Time courses of UCP mRNA and protein expression in rat epididymal adipocytes. The UCP mRNA and protein levels were measured in rat adipocytes cultured for 0 to 8 h in medium containing 20 mM glucose with or without 0.1 μM insulin. The relative mRNA and protein levels were normalized by those at time zero. Means±SEM of six different experiments. Two-way ANOVA for UCP-2 mRNA : T (time), $P<0.001$, Ins (insulin), $P<0.001$; UCP-3 mRNA : T, $P<0.001$, Ins, $P<0.05$; UCP-3 protein : T, $P<0.001$, Ins, $P<0.05$, respectively.

T3 or epinephrine dose-dependent regulation of UCP mRNA and protein in rat epididymal adipocytes

The does-dependent regulation of UCP mRNA and protein by T3 or epinephrine are shown in Fig. 4 or 5. The addition of T3 or epinephrine to the culture medium caused marked dose-dependent increases in the expression of UCP-2 and -3 mRNAs. The expression of UCP-2 and -3 proteins by T3 or epinephrine was about parallel to their mRNA expression.

Time courses of UCP mRNAs and proteins expression by T3 or epinephrine in rat epididymal adipocytes

The time courses of UCP mRNAs and proteins expression were measured after addition of T3 or epinephrine (Fig. 6). The mRNA and protein expression of UCPs in epididymal adipocytes reached each a maximal level at 2 h after the treatment with T3 or epinephrine. The expression of UCP mRNAs

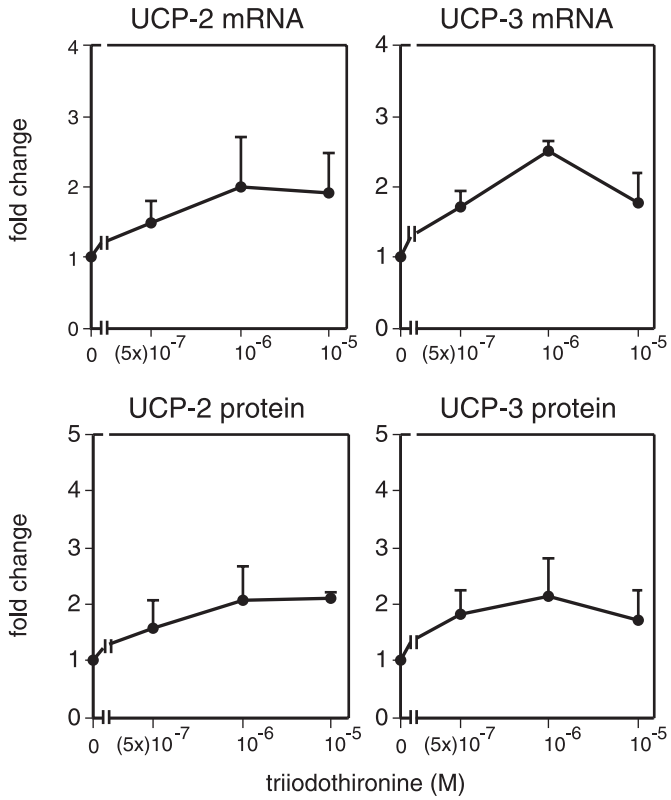

Fig. 4. T3 dose-dependent regulation of UCP mRNA and protein. The rat epididymal adipocytes were cultured for 4 h in medium containing 5 mM glucose with or without 0.1 μM insulin, and the UCP mRNA and protein levels were measured. The relative mRNA and protein levels were normalized by those with no addition. Means±SEM of six different experiments. Two-way ANOVA for UCP-2, -3 mRNA : T3, $P<0.05$; UCP-2, -3 protein : T3, $P<0.05$, respectively.

reached a peak earlier and was significantly higher with T3 or epinephrine than without the treatment (insulin＋glucose). T3 or epinephrine addition to the culture medium stimulated the expression of UCPs only in the presence of glucose (data not shown). Thus, UCP-2 and -3 protein expression was stimulated by T3 or epinephrine.

Effects of fatty acids on UCP mRNA expression in rat epididymal adipocytes

Equivalent amounts of fatty acids or triacylglycerols were added to the cell culture medium, and their effects on UCP mRNA expression were examined. The mRNA expression of UCP-2 and -3 was significantly increased by unsaturated fatty acids such as oleic acid (18:1), linoleic acid (18:2) or arachidonic acid (20:4), whereas their expression was not increased by saturated fatty acids such as palmitic acid (16:0) (Fig. 7). The UCP mRNA expression was also increased by triolein or trilinolein. The UCP protein expression levels were parallel with the levels of UCP mRNA expression (data not

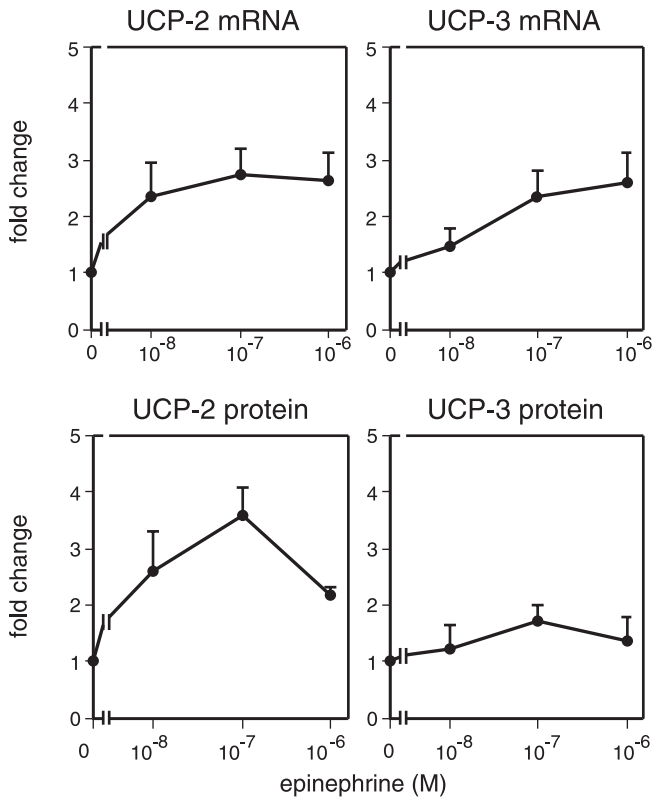

Fig. 5. Epinephrine dose-dependent regulation of UCP mRNA and protein. The rat epididymal adipocytes were cultured for 4 h in medium containing 5 mM glucose with or without 0.1 μM insulin, and the UCP mRNA and protein levels were measured. The relative mRNA and protein levels were normalized by those with no addition. Means±SEM of six different experiments. Two-way ANOVA for UCP-2, -3 mRNA : epinephrine : $P < 0.001$, I ; UCP-2, -3 protein : epinephrine, $P < 0.001$, respectively.

shown).

Discussion

In the present study, UCP-2, -3 mRNA expression was stimulated dose-dependently by glucose and reached peaks 4 h after the treatment in primary cultured epididymal adipocytes, and then decreased. The UCP protein induction reached a maximal level at 4 h. The UCP-2 and -3 mRNA and protein expression was stimulated in the presence of insulin. Moreover, the induction of UCPs mRNA and protein was accelerated by T3 or epinephrine, and reached a maximum at 2 h. Thus, the induction of UCPs mRNA and protein was rapid.

We previously investigated the time courses of UCP-1, -2 and -3 mRNA induction after refeeding a fat-free or corn oil diet to food-deprived rats[27]. The mRNA induction of the UCPs in white and brown adipose tissues was already elevated 1 h after the refeeding, and reached a maximal level at about 1 to 2

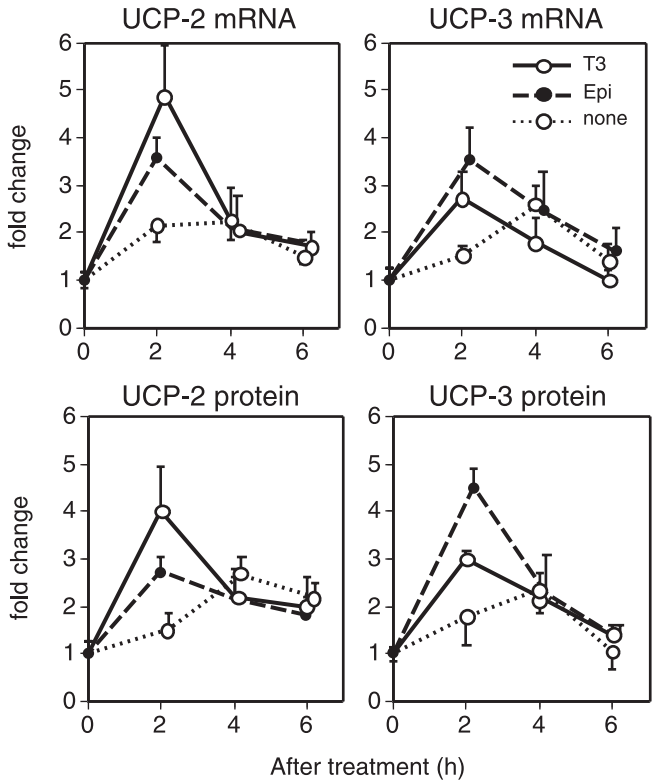

Fig. 6. Effects of T3 and epinephrine on time courses of UCP mRNA and protein expression. Time courses of UCP mRNA and protein levels of adipocytes cultured for 6 h in medium containing 20 mM glucose were measured. When included, 0.1 μM T3 or 1 μM epinephrine (Epi) was added. The relative mRNA and protein levels were normalized by those with no hormone at time zero. Means±SEM of six different experiments. Two-way ANOVA for UCP-2 mRNA : T (time), $P<0.001$, T3, Epi, $P<0.01$, T×T3, T×Epi, $P<0.01$; UCP-3 mRNA : T, $P<0.001$, T3, Epi, $P<0.01$; UCP-2 protein : T, $P<0.001$, T3, Epi, $P<0.05$, T×T3, T×Epi, $P<0.01$; UCP-3 protein : T, $P<0.001$, T3, Epi, $P<0.05$, T×T3, T×Epi, $P<0.001$, respectively.

h. Moreover, the induction of UCP-2 mRNA was earlier and significantly higher at the peak in rats fed the corn oil diet than in those fed the fat-free diet[27].

N-6 polyunsaturated fatty acids induced a 3-fold rise in UCP-2 expression in primary cultures of human muscle cells, whereas n-3 polyunsaturated fatty acids did not[28]. Increases in free fatty acids resulting from fasting or a high-fat diet lead to stimulation of UCP-2 expression in adipose tissue and muscle[29]. In the present study, the UCP mRNA expression was significantly elevated by unsaturated fatty acids but not by saturated fatty acids. Thus, the effects of different types of fatty acid on the stimulation of UCP expression were not always similar and still remain to be clarified.

Barbe *et al.* reported that UCP-2 mRNA expression in human subcutaneous adipose tissue was does-dependently increased by T3[30]. Our results coincide with those results. Thyroid hormone is a key regulator of thermogenesis and basal metabolic rate, and works through mechanisms largely involving

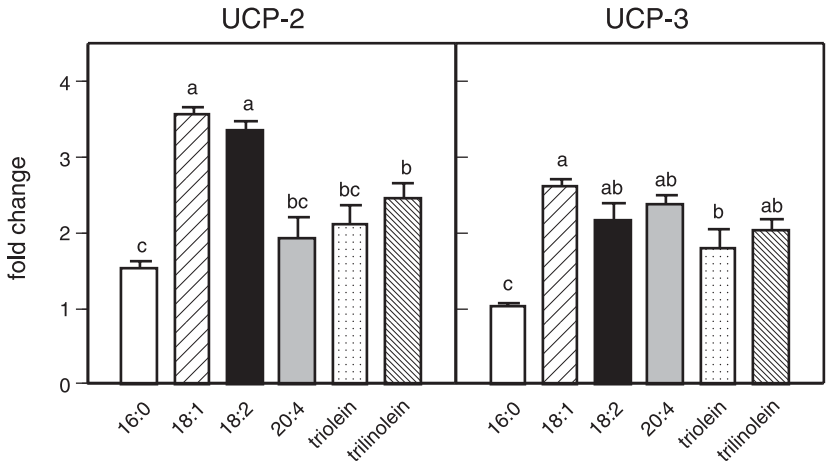

Fig. 7. Effects of fatty acids on UCP mRNA expression in rat epididymal adipocytes. The adipocytes were cultured for 4 h in medium containing 5 mM glucose (Glu) with 0.1 μM insulin (Ins), and the UCP mRNA levels were measured. When included, 0.1 mM palmitic acid (16:0), oleic acid (18:1), linoleic acid (18:2), arachidonic acid (20:4), 0.03 mM triolein or trilinolein was added. The relative mRNA levels were normalized by those obtained with Glu＋Ins. Means±SEM of six different experiments. One-way ANOVA for UCP-2 and UCP-3 : fatty acid, $P < 0.001$.

transcriptional regulation of gene expression[31, 32]. Although UCP-3 expression was stimulated by epinephrine, UCP-2 expression was stimulated by T3, glucose or insulin, similarly to lipogenic enzymes[33] rather than by epinephrine.

Medvedev *et al.* reported that the region from -86 to -44 of the UCP-2 promoter is composed of three overlapping putative elements, Sp1, SRE, and a double E-box[34]. We found that common GC-rich sequences were responsible for glucose/insulin-stimulation in the proximal promoter regions from -57 to -35 of the fatty acid synthase gene, -64 to -41 of the ATP citrate-lyase gene, and -101 to -83 of the leptin gene in white adipose tissue of rat. These results obtained using transfected reporter genes are coincident with the effects of glucose, insulin, T3 and epinephrine to stimulate the endogenous UCP genes described here. Thus, UCP-2 expression appears to be stimulated by energy sources such as glucose and fat, and by regulators of thermogenesis and basal metabolic rate such as T3 and insulin, unlike UCP-3 expression in rat epididymal adipose tissue. The regulation of UCPs gene expression of mesenteric adipose tissue will be elucidated in the future studies.

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